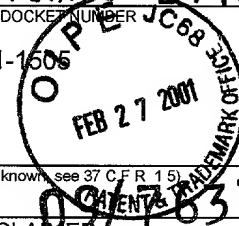


**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

24741-1505



U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.51)

INTERNATIONAL APPLICATION NO.  
PCT/EP99/06280

INTERNATIONAL FILING DATE  
26 August 1999 (26.08.99)

PRIORITY DATE CLAIMED  
27 August 1998 (27.08.98)

## TITLE OF INVENTION

LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC ACID FOR PRODUCING VACCINES

## APPLICANT(S) FOR DO/EO/US

Jan SIMON, Stefan MARTIN, Christian TERMEER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371©(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371 ©(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371©(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371©(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371©(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371©(5)).

## Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO. (If known, see 37 CFR 1.50) 09/674,019 <b>09/763794</b>	INTERNATIONAL APPLICATION NO. PCT/EP99/02592	ATTORNEY'S DOCKET NUMBER 37637-0002US
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17. ☐ The following fees are submitted:**CALCULATIONS**

PTO USE ONLY

**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Neither international preliminary examination fee (CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . \$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =** \$860

Surcharge of \$130.00 for furnishing the oath or declaration later than 20 \_\_\_\_ 30 \_\_\_\_ months from the earliest claimed priority date (37 CFR 1.492(e)) \$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	26 -20 = 6	6	X \$18.00	\$108.00	
Independent Claims	4-3 = 1	1	X \$80.00	\$80.00	

MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00 \$

**TOTAL OF ABOVE CALCULATIONS =** \$954

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). \$477

**SUBTOTAL =** \$477

Processing fee of \$130.00 for furnishing English translation later the 20 \_\_\_\_ 30 \_\_\_\_ months from the earliest claimed priority date (37 CFR 1.492(f)). + \$

**TOTAL NATIONAL FEE =** \$477

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property + \$

**TOTAL FEES ENCLOSED =** \$477

Amount to be:  
refunded \$

charged: \$


- a. ☒ A check in the amount of \$477 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 08-1641 in the amount of \$\_\_\_\_\_ to the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-1641. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

**Customer ID No. 26633**

Patricia D. Granados  
HELLER ERHMAN WHITE MCAULIFFE  
815 Connecticut Avenue, NW, Suite 200  
Washington, DC 20006-4004  
Tel: (202) 263-8900  
Fax: (202) 785-8877

  
SIGNATURE  
Patricia D. Granados  
NAME

33,683  
REGISTRATION NUMBER

#4/B

**Rec'd PCT/PTO 12 JUL 2001**  
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Atty. Docket No: 24741-1505**

*In re* patent application of  
SIMON, Jan *et al.*

Serial No.: 09/763,794

Group Art Unit: Unassigned

Filed: July 12, 2001

Examiner: Unassigned

For: LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC ACID FOR  
PRODUCING VACCINES

**AMENDMENT IN RESPONSE TO NOTICE**  
**UNDER 37 CFR §§ 1.821 – 1.825**

Director of Patents  
Washington, D.C. 20231  
**Box SEQUENCE**

Sir:

In response to the Notification to Comply With Requirements for Applications containing Sequence Disclosures mailed May 14, 2001, please amend the application as follows:

**IN THE SPECIFICATION:**

**Page 18, paragraph 6, line 25:** Please amend as follows:

Mice (C57/BL6, female, 6-12 weeks, 60 animals, 5 mice per experimental group, 20 g per mouse) were injected with dendritic cells which had previously been treated with a synthetically prepared protein having a defined amino acid sequence, i.e. a (SIINFEK\*L (SEQ ID NO: 1), SIIK\*FEKL (SEQ ID NO: 2); \* = TNP lysine) peptide. The peptide is selected such that it can react directly with the MHC molecule antigen-binding site on the cell surface of antigen-presenting cells; this means that it is presented on the surface of dendritic cells immediately after they have been added. The hapten trinitrophenyl (TNP), which constitutes the antigenic determinant, is in turn coupled to this peptide. This means that a T cell which is activated by the trinitrophenyl-coupled peptide which is presented by the dendritic cell becomes reactive to trinitrophenyl or chemicals which are structurally homologous to trinitrophenyl, such

0963794.01001

as the trinitrochlorobenzene (TNCB) used during the challenge which is implemented below. Accordingly, in this system, the strength of the trinitrophenyl-specific T cell reaction depends on the degree to which the dendritic cells are activated and/or the efficiency with which the trinitrophenyl-coupled peptide is presented to the T cells by the dendritic cells. The method is a recognized model for the in-vivo investigation of mechanisms which play a role during T cell activation.

Page 29, paragraph 3, line 11: Please amend as follows:

Antigen name:	Peptide sequence:	Target cell:
MELAN-1/MART-1	EAAGIGILTV (SEQ ID NO: 3)	human melanoma
Tyrosinase	AFLPWHRLFL (SEQ ID NO: 4)	human melanoma
GP-33	KAVYNFATM (SEQ ID NO: 5)	LCM virus

Page 34, at the end of the specification, insert the Sequence Listing submitted concurrently herewith.

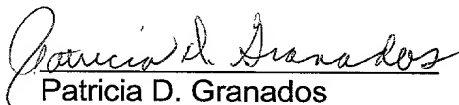
#### REMARKS

Applicants submit this Amendment to insert the required SEQ ID NOS of the Sequence Listing filed concurrently herewith and to indicate the insertion point for the Sequence Listing.

Applicants respectfully request examination on the merits of this application.

Respectfully submitted,

July 12, 2001  
Date

  
Patricia D. Granados  
Reg. No. 33,683

**Heller Ehrman White & McAuliffe LLP**  
1666 K Street, NW, Suite 300  
Washington, DC 20006-1229  
Telephone: (202) 912-2000  
Facsimile: (202) 912-2020

PATENT TRADEMARK OFFICE



26633

**Amendments to specification with brackets and underlining**

**Page 18, paragraph 6, line 25:** Please amend as follows:

Mice (C57/BL6, female, 6-12 weeks, 60 animals, 5 mice per experimental group, 20 g per mouse) were injected with dendritic cells which had previously been treated with a synthetically prepared protein having a defined amino acid sequence, i.e. a (SIINFEK\*L (SEQ ID NO: 1), SIIK\*FEKL (SEQ ID NO: 2); \* = TNP lysine) peptide. The peptide is selected such that it can react directly with the MHC molecule antigen-binding site on the cell surface of antigen-presenting cells; this means that it is presented on the surface of dendritic cells immediately after they have been added. The hapten trinitrophenyl (TNP), which constitutes the antigenic determinant, is in turn coupled to this peptide. This means that a T cell which is activated by the trinitrophenyl-coupled peptide which is presented by the dendritic cell becomes reactive to trinitrophenyl or chemicals which are structurally homologous to trinitrophenyl, such as the trinitrochlorobenzene (TNCB) used during the challenge which is implemented below. Accordingly, in this system, the strength of the trinitrophenyl-specific T cell reaction depends on the degree to which the dendritic cells are activated and/or the efficiency with which the trinitrophenyl-coupled peptide is presented to the T cells by the dendritic cells. The method is a recognized model for the in-vivo investigation of mechanisms which play a role during T cell activation.

**Page 29, paragraph 3, line 11:** Please amend as follows:

Antigen name:	Peptide sequence:	Target cell:
MELAN-1/MART-1	EAAGIGILTV ( <u>SEQ ID NO: 3</u> )	human melanoma
Tyrosinase	AFLPWHRLFL ( <u>SEQ ID NO: 4</u> )	human melanoma
GP-33	KAVYNFATM ( <u>SEQ ID NO: 5</u> )	LCM virus

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Atty. Docket No: 24741-1505**

*In re* patent application of  
SIMON, Jan *et al.*

Serial No.: 09/763,794

Group Art Unit: Unassigned

Filed: July 12, 2001

Examiner: Unassigned

For: LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC ACID FOR  
PRODUCING VACCINES

**CHANGE OF ADDRESS**


Director of Patents  
Washington, D.C. 20231

Sir:

Applicant respectfully requests that the official correspondence address for the above captioned application be changed to Patricia D. Granados and the attorneys of Heller Ehrman White & McAuliffe LLP, located at 1666 K Street, N.W., Suite 300, Washington, D.C. 20006. Please direct all written communications to Patricia D. Granados at the above mentioned address, and direct all telephone communications to (202) 912-2000.

Respectfully submitted,

July 12, 2001  
Date

  
Patricia D. Granados  
Registration No. 33,683

**Heller Ehrman White & McAuliffe LLP**  
1666 K Street, N.W.  
Suite 300  
Washington, D.C. 20006  
Telephone: (202) 912-2000  
Facsimile: (202) 912-2020



26633

PATENT TRADEMARK OFFICE

# SEQUENCE LISTING

<110> SIMON, Jan  
MARTIN, Stefan  
TERMEER, Christian

<120> LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC ACID FOR PRODUCING VACCINES

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09/763794

24741-1505

JC03 Rec'd PCT/PTO 27 FEB 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: 24741-1505

Applicant:

Simon, Jan *et al.*

Appl. No.: PCT/EP99/06280

Filing Date: Herewith

Examiner: Unassigned

Art Unit: Unassigned

Title: LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC  
ACID FOR PRODUCING VACCINES

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents

Box *PCT*

Washington, D.C. 20231

Sir:

Prior to examination on the merits, Applicants respectfully request that the  
above-identified application be amended as follows:

**IN THE CLAIMS**

Please cancel claims 1 through 33 without prejudice or disclaimer.

Please add the following new claims:

34. (New) A process for concentrating dendritic cells, comprising:

- a) isolating mononuclear cells from blood;
- b) concentrating mononuclear cells possessing CD14 surface

marker;

- c) culturing the cells of step b) in a medium containing cytokines GM-CSF and IL-4; and

- d) culturing the cells of step c) together with hyaluronic acid fragments, wherein said hyaluronic acid fragments possess from 1 to 50 hyaluronic acid basic building blocks, in order to cause the cells to mature irreversibly into dendritic cells, the basic building block being an aminodisaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by a  $\beta$ 1-3 glycosidic bond.

35. (New) The process of claim 34, wherein the hyaluronic acid fragments of step d) are modified.

36. (New) The process of claim 34, wherein the mononuclear cells are isolated from a leukocyte concentrate using a density gradient.

37. (New) The process of claim 36, wherein the density gradient is a Ficoll density gradient.

38. (New) The process of claim 34, wherein the cells possessing the CD14 surface marker are concentrated using at least one antibody that is directed against the CD14 surface marker.

39. (New) The process of claim 34, wherein the cells possessing the CD14 surface marker are cultured in a medium that contains GM-CSF at a concentration from 5,000 to 10,000 U/mL and IL-4 at a concentration from 100 to 1,000 U/mL.

40. (New) The process of claim 34, wherein the hyaluronic acid fragments each contain from 1 to 10 aminodisaccharides.

41. (New) The process of claim 34, wherein the cells possessing the CD14 surface marker are cultured for 72 hours to 7 days in a medium containing GM-CSF and IL-4.

42. (New) The process of claim 34, wherein the cells in step d) are cultured together with hyaluronic acid fragments for at least 48 hours.

43. (New) The process of claim 35, wherein the hyaluronic acid fragments are chemically modified.

44. (New) A vaccine comprising an antigen or peptide and a low molecular weight hyaluronic acid fragment consisting of 1 to 50 basic units,  
wherein the basic unit is an aminodisaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine linked by a  $\beta$ 1-3 glycosidic bond.

45. (New) The vaccine of claim 44 further comprising a carrier system.

46. (New) The vaccine of claim 44, wherein the hyaluronic acid fragments are modified.

47. (New) The vaccine of claim 46, wherein the hyaluronic acid fragments are chemically modified.

48. (New) The vaccine of claim 44, which is formulated for subcutaneous, intracutaneous or intravenous administration.

49 (New) The vaccine of claim 44, wherein the vaccine comprises two separate formulations, wherein one formulation comprises an antigen or peptide and the second formulation comprises the low molecular weight hyaluronic acid fragment.

50. (New) The composition of claim 49, which further comprises a carrier system.

51. (New) The composition of claim 49, wherein the low molecular weight hyaluronic acid fragment is modified.

52. (New) The composition according to claim 51, wherein the low molecular weight hyaluronic acid fragment is chemically modified.

53. (New) The vaccine of claim 44, wherein the low molecular weight hyaluronic acid fragment is coupled to the antigen or peptide.

54. (New) The composition of claim 53, wherein the low molecular weight hyaluronic acid fragment is modified.

55. (New) The composition of claim 54, wherein the low molecular weight hyaluronic acid fragment is chemically modified.

56. (New) The composition of claim 49, wherein the peptide or the antigen is present as a separate molecule in a microsphere.

57. (New) A vaccine comprising dendritic cells that have been cultured with a low molecular weight hyaluronic acid fragment consisting of 1 to 50 basic units,

wherein the basic unit is an aminodisaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine linked by a  $\beta$ 1-3 glycosidic bond.

58. (New) The vaccine of claim 57, which further comprises an antigen or peptide.

59. (New) A method of maturing dendritic cells comprising culturing the dendritic cells with hyaluronic acid fragments consisting of 1 to 50 basic units, wherein the

basic unit is an aminodisaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine linked by a  $\beta$ 1-3 glycosidic bond.

### REMARKS

Applicants respectfully request that the foregoing amendments be made prior to examination of the present application. Claims 1 to 33 have been canceled without disclaimer or prejudice. New claims 34 to 59 have been added and are pending. No new matter has been added with the new claims, which are supported by the original claims but have been re-drafted for U.S. practice.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below. Examination on the merits is respectfully requested.

Respectfully submitted,

Date

February 27, 2001

By

Patricia Granados

Patricia Granados  
Attorney for Applicant  
Registration No. 33,683

Heller Ehrman White & McAuliffe  
815 Connecticut Avenue N.W.  
Washington, D.C. 20006  
Telephone: (202) 263-8969  
Facsimile: (202) 785-8877

09/763794

26 August 1999

Albert Ludwigs University Clinical Centre, Freiburg  
Hugstetter Straße  
79106 Freiburg

5

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**Low molecular weight fragments of hyaluronic  
acid for producing vaccines**

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10 The invention relates to the use of low  
molecular weight fragments of hyaluronic acid in  
immunotherapy, in particular for producing compositions  
which can be used in immunotherapy. Within the context  
of this application, such compositions, which can be  
employed in immunotherapy, are referred to generally as  
15 "vaccines".

The preparation of dendritic cells (DC) is  
gaining ever increasing importance in the therapy of a  
variety of diseases. The dendritic cells can be used to  
prepare highly active immunomodulators which can be  
20 loaded with a variety of antigens, in particular tumour  
antigens, virus peptides or compounds having an  
allergenic effect. These cells are then employed, in  
adoptive immunotherapy, in patients with tumours or who  
are suffering from virus diseases or allergies. There  
is therefore a substantial need in adoptive  
25 immunotherapy for dendritic cells, which are preferably  
to be prepared using standardized, reproducible and  
inexpensive methods. These methods have to be carried  
out under GMP/GLP conditions.

30 A variety of methods for producing dendritic  
cells from stem cells are known from the prior art.

Haematopoietic stem cells which possess the  
CD34 surface marker can be isolated from the bone  
marrow or peripheral blood of chemotherapy patients who  
35 have been treated with granulocyte colony-stimulating  
factor (G-CSF). These stem cells are cultured in the  
added presence of a variety of cytokines and the

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dendritic cells are obtained after a relatively long period of culture.

By now, methods have become available for generating dendritic cells in vitro in large numbers from precursor cells present in peripheral blood (J. Immunol. Med. 1996, 196: 137-51; Exp. Hematology 1997, 25: 232-36; Ann. Surgery 1997, 226: 6-16).

While a study carried out on melanoma patients using immature dendritic cells of the prior art shows response rates in 5 out of 16 patients (Nature Medicine 1998, 4: 328-32), it was possible to induce T cells having specificity for the melanoma antigen coupled to the dendritic cells in all the patients. However, there is the problem that tolerance to the injected peptide can be induced when the quantity of peptide is too low or when peptides have been modified with regard to their binding strength (J. Immunol. 1998, 160: 4449-56). As far as tumour therapy is concerned, this situation would be fatal; instead of immunizing the patient, this situation would cause the induced tumour antigens to be tolerated. Investigations carried out by the inventors have shown that the effect cannot be circumvented by injecting a relatively large quantity of dendritic cells, either. On the contrary, the degree of activation or maturation of the dendritic cells is a crucial factor in determining whether the use of these cells is likely to be successful.

A method for maturing dendritic cells using monocyte conditioned medium (MCM), tumour necrosis factor alpha (TNF $\alpha$ ) or CD40 ligation is known from the prior art (J. Immunol. Methods 1996, 196: 121-135; J. Exp. Med. 1997, 185: 341-349; Blood 1998, 91: 4652-4661). In these methods, monocytes are isolated from peripheral blood and cultured with GM-CSF and IL-4. In order to complete the maturation of the cells into dendritic cells, additional cytokines have to be added at the end of the differentiation phase.

However, the methods which are known from the prior art suffer from a variety of disadvantages. The

relatively long culture period is not suitable for hospital use. In addition, the high costs of the cytokines and the batch-dependence of the cytokines employed can be disadvantageous.

5 In the case of conventional vaccinations with antigens or peptides, there is also the problem that the immune response is frequently too weak or that, as mentioned above, the preparation of mature dendritic cells, which might be able to induce a better immune  
10 response, is too elaborate, too expensive and not sufficiently reproducible. In addition, it has been found, when vaccinating tumour patients, that, while immune responses can be elicited with a local antigen injection, these responses remain, to a large extent,  
15 restricted to the injected limb or even only to the closest lymph node station. There is, therefore, a need for a vaccination strategy, particularly for tumour patients, in whom the antigen can also be administered systemically in order to induce a generalized immune  
20 response. The most obvious solution to this problem, namely that of administering an intravenous dose of peptide and a suitable adjuvant, suffers from the disadvantage that, as a result of the dilution effect in the peripheral blood, the adjuvant becomes inactive  
25 in a very short period of time.

These problems can be solved on the basis of the surprising finding that low molecular weight hyaluronic acid fragments can be employed advantageously in different ways in immunotherapy, in  
30 particular for vaccinating tumour patients.

The present invention therefore makes available, in a general manner, the use of low molecular weight hyaluronic acid fragments, which can be suitably modified chemically, where appropriate, for producing a  
35 vaccine, in particular for vaccination by means of subcutaneous (s.c.), intracutaneous (i.c.) and intravenous (i.v.) vaccination, in particular for treating tumour patients.

In a first embodiment, the present invention

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makes available a process for concentrating mature dendritic cells, which process comprises the following steps:

- a) mononuclear cells are isolated from blood,
- 5 b) cells which possess the CD14 surface marker are concentrated,
- c) the cells which possess the CD14 surface marker are cultured in a medium which contains the cytokines GM-CSF and IL-4, and
- 10 d) the cells obtained in step c) are cultured together with hyaluronic acid fragments, which can be suitably modified, where appropriate, in order to induce the cells to mature into dendritic cells.

15 The invention likewise relates to vaccines which comprise the appropriately matured dendritic cells and to the use of the dendritic cells which have been matured with hyaluronic acid fragments for producing such vaccines.

20 In a further embodiment, the invention relates to vaccines which comprise low molecular weight hyaluronic acid fragments, which may be chemically modified, where appropriate, as adjuvant.

In a further embodiment, the invention relates  
25 to systems which contain an antigen or peptide, where appropriate together with a carrier system and, coupled to it, a low molecular weight hyaluronic acid fragment which can be suitably modified, where appropriate. In a further embodiment, the invention relates to vaccines  
30 which comprise an antigen or peptide which has been modified in this way with a hyaluronic acid fragment.

Particularly preferably, the vaccines according to the invention are used for subcutaneous (s.c.), intracutaneous (i.c.) or intravenous (i.v.) vaccina-  
35 tion, in particular of tumour patients.

In that which follows, the first embodiment of the invention, namely the process for concentrating mature dendritic cells with hyaluronic acid fragments, which can be suitably modified, where appropriate, is

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described first of all, as are the corresponding vaccines which comprise these mature dendritic cells.

Mononuclear cells can be isolated from blood using a density gradient, with a leukocyte concentrate  
5 being fractionated through a Ficoll density gradient in a preferred embodiment.

In the second step of the process, the cells possessing the CD14 surface marker are concentrated, preferably using at least one antibody which is  
10 directed against the CD14 surface marker. Magnet-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) can be employed in this connection. A simpler, but not so efficient method is that of enrichment by means of adhesion to plastic.

15 The cells possessing the CD14 surface marker are then cultured in a medium which contains GM-CSF at a concentration of from 5 000 to 10 000 U/ml and IL-4 at a concentration of from 100 to 1 000 U/ml. Where appropriate, other suitable cytokines can also be  
20 added.

Finally, the cells which are obtained in the culturing step are cultured with hyaluronic acid fragments, with these fragments possessing from 1 to 50 basic building blocks of hyaluronic acid. A basic  
25 building block consists of an aminodisaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by a  $\beta$ 1-3 glycosidic bond. Preference is given to using hyaluronic acid fragments which each contain from 1 to 10 basic build-  
30 ing blocks.

The cells possessing the CD14 surface marker are cultured in a medium containing GM-CSF and IL-4, preferably for a period of from at least 72 h up to 7 days. The cells are then cultured, in step d), with  
35 hyaluronic acid fragments for at least 48 hours. The hyaluronic acid fragments are preferably present at a concentration of from 1 to 50  $\mu$ g/ml, and particularly preferably at a concentration of from 10 to 30  $\mu$ g/ml.

Consequently, the present invention relates, in

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F02F20/462960

one embodiment, to the use of low molecular weight hyaluronic acid fragments for maturing dendritic cells.

5 The process according to the invention can be used to prepare and/or concentrate dendritic cells. The dendritic cells are antigen-presenting in cells which are specialized for initiating the primary immune response. They assume different functions in the different stages of their development. In the immature state, dendritic cells are very efficient in processing  
10 native protein antigens for the MHC class II route. By comparison, mature dendritic cells are less suitable for taking up new proteins for presentation; on the other hand, they are much better at stimulating the growth and differentiation of resting CD4<sup>+</sup> and CD8<sup>+</sup> T  
15 cells.

In vivo, maturation of the dendritic cells begins when the immature dendritic cells migrate from the sites of antigen uptake to the T cell regions of the lymphoid organs. In vitro, it is possible to  
20 observe the maturation in cultures of freshly isolated dendritic cells. The maturation of the dendritic cells is also reflected in changes in morphology and phenotype. Dendritic cells are usually characterized and differentiated by detecting a variety of surface  
25 markers.

Because of their natural functions, dendritic cells are particularly suitable for being used as a natural adjuvant in vaccination and immunotherapy, in particular in tumour therapy. In this connection, it is  
30 of crucial importance, when the cells are being employed to achieve the sought-after therapy, to use fully matured DCs, which are unable to differentiate back, once again, into macrophage-like precursors following reinfusion into the patient.

35 According to the current state of knowledge, it is assumed that the dendritic cells take up the antigen (for example a tumour antigen) when they are in the immature state and then process the antigen. In connection with doing this, the dendritic cells mature and

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migrate to the T-cell-rich regions of the secondary lymphoid organs. Mature dendritic cells express large numbers of MHC molecules, costimulatory molecules and adhesion molecules on their surfaces, which molecules enable the dendritic cells to interact with the T cells (T cell clustering). Costimulatory signals are transmitted by way of B7-CD28 and CD40-CD40-ligand interactions, and are amplified by the production of cytokines, such as IFN- $\alpha$ , IFN- $\gamma$  and IL-12, which, as is known, promote cell-mediated immunity.

According to the current state of knowledge, it appears to be essential for specialized antigen-presenting cells to be present for inducing a primary T cell response, since presenting antigens to T cells in the absence of a second signal (costimulation) may either cause the T cells to die or produce antigen-specific tolerance.

The advantages of the process according to the invention are therefore to be seen, in particular, in the fact that the dendritic cells can be prepared relatively simply. In addition, it is only necessary to use relatively low concentrations of expensive cytokines. The process according to the invention makes it possible to prepare a cell population which consists to a very large extent of dendritic cells, with these cells being in a relatively uniform (synchronous) state of maturity. When the dendritic cells are to be used for presenting antigens, the appropriate antigens can be added to the dendritic cells at a suitable time and the antigens can then be processed by the dendritic cells during the course of the maturation process. The antigens can be added as proteins, in the form of killed cells or cell preparations, or in the form of recombinantly modified cells. When the dendritic cells are in the desired state, they can then be used for the therapy.

The process according to the invention is suitable for preparing autologous dendritic cells. The term "autologous" means that the starting cells are

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taken from a donor (patient). These cells are then treated in accordance with the invention in vitro in order to obtain dendritic cells. The concentrated dendritic cells are then, where appropriate after  
5 contact with the antigen, readministered to the same donor from whom they were originally derived.

Alternatively, the process can also be used for preparing "allogenic" dendritic cells. In this case, the cells are prepared from a donor, or else from  
10 stored blood, which can be obtained from blood donor centres, and then readministered, in this case to other recipients. This makes it possible to prepare standardized dendritic cells which are, for example, optimally loaded with a tumour antigen. In this way, it  
15 is possible to increase cellular immunity efficiently, in particular in association with viral infection (HIV).

According to the invention, fragments of hyaluronic acid (HA), which may, where appropriate, be  
20 suitably modified, are employed for maturing the dendritic cells. Hyaluronic acid is a macromolecular polysaccharide which, as an endogenous substance, is used, first and foremost, in the corium for storing water. Hyaluronic acid is produced, in particular, by  
25 keratinocytes in the basal layers of the epidermis and by fibroblasts present in the subcutaneous connective tissue. Hyaluronic acid is also present in the vitreous humour of the eye and in the synovial fluid of the joints and is a constituent of connective tissue. At  
30 low concentrations, hyaluronic acid forms a highly viscous aqueous solution. Hyaluronic acid is a high molecular weight compound having a molecular weight of between 50 000 daltons and several million daltons. The basic building block of hyaluronic acid is an amino-  
35 disaccharide which consists of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by a  $\beta$ 1-3 glycosidic bond. This basic building block is linked to the next unit by a  $\beta$ 1-4 glycosidic bond. This unbranched hyaluronic acid chain consists of from about

2 000 to 10 000 of such basic units. Hyaluronidases hydrolyse the  $\beta$ -glucosidic bonds and the hyaluronic acid is in this way broken down into smaller fragments. According to the invention, preference is given to using hyaluronidase from bull testes or the hyaluronidase which is isolated from *Streptococcus hyaluronicus*. The hyaluronic acid fragments which are employed in accordance with the invention are preferably first of all comminuted mechanically by shearing forces and/or ultrasonication, after which the polysaccharides are subjected to further degradation using a suitable hyaluronidase. The desired fragments, preferably consisting of from 1 to 50 basic units, are then isolated using suitable separation methods.

The hyaluronic acid fragments can be suitably modified chemically either before or after isolation. Preference is given to modifying them after isolation. Examples of modifications which may be mentioned are esterification, salt formation, amidation, reduction of an acid group to the aldehyde or alcohol, or elimination of an acid group. Salts and esters of the hyaluronic acid fragments, for example alkali metal salts and alkaline earth metal salts, and  $C_1$ - $C_{10}$ -alkyl esters, preferably  $C_1$ - $C_4$ -alkyl esters, are preferred. Other chemical modifications are also possible and can be effected by a skilled person, without difficulty, on the basis of his specialist knowledge.

The present invention is explained in more detail with the aid of the examples which are described below. In carrying out the examples, particular attention was paid to avoiding contamination of the reagents with lipopolysaccharides (LPS). Lipopolysaccharides are constituents of the cell walls of Gram-negative bacteria. Even the smallest contamination with lipopolysaccharides is sufficient to activate monocytes or  $CD14^+$  stem cells present in the peripheral blood irreversibly. Care was therefore taken, in the experiments carried out in accordance with the invention, to keep below a threshold value of 0.01 ng of lipo-

polysaccharide/ml, below which value lipopolysaccharide has been found by experience not to have any effect on monocytes, CD14<sup>+</sup> stem cells or dendritic cells.

5 **Example 1: Cell isolation**

a) **Isolating mononuclear cells (PBMC)**

10 A leukocyte concentrate (buffy coat) from a healthy human donor was fractionated by centrifuging it through a density gradient containing Ficoll-Hyperpaque plus (Pharmacia, Uppsala, Sweden). Under these conditions, the mononuclear cells (PBMC) come to lie in the interphase zone of the gradient while red blood cells (erythrocytes) and neutrophilic granulocytes are to be found below the gradient, and are subsequently  
15 discarded. While Ficoll-Hyperpaque plus is a mixture of macromolecular sugars of cellular origin, its LPS content is tested by the manufacturer (endotoxin content < 0.001 ng/ml). This value was confirmed in our own tests.

20 b) **Labelling the CD14-positive cells**

In order to purify the monocytes still further, the PBMC were incubated for 45 min with anti-CD14 Mab. After that, they were washed with PBS and the cell pellet was resuspended in 2 ml of MACS buffer. MACS®  
25 (Miltenyi, Biotech, Bergisch Gladbach) = magnetic-activated cell sorting; a method for purifying cells using antibodies which are bound to metal beads; labelled cells remain suspended in the magnetic column matrix. [PBS w/o Ca<sup>2+</sup>/Mg<sup>2+</sup> (Gibco) containing 5 mM  
30 ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA); pH adjusted to 7.2 by adding HCl]. Adding EDTA prevents the cells from clumping, something which is an important prerequisite for the purification in the column. The cells were now incubated, under the  
35 same conditions, with 50 µl of goat anti-mouse IgG microbeads and then washed with MACS buffer, centrifuged and resuspended in 5 ml of MACS buffer.

c) Concentrating the CD14-positive cells using MACS

The concentration took place at 4°C. The VS+® column was inserted in the MACS magnets (both from Miltenyi) and first of all washed with 5 ml of MACS buffer. In order to obtain a single-cell suspension, the cells were passed through a cell strainer (30 µm mesh width; Miltenyi) and loaded onto the column. The negative fraction, consisting of unlabelled cells which were not retained magnetically in the column matrix, passed through and were discarded. The column was washed a further two times with 5 ml of MACS buffer on each occasion in order to obtain a positive fraction which was as pure as possible. The column was then removed from the magnets and the cells which were retained in the matrix specifically by means of microbeads were washed, under pressure and using 5 ml of buffer, into a sterile tube. The cells which were obtained were counted in a Neubauer chamber. As a rule, it was possible, using this method, to obtain  $3-5 \times 10^7$  CD14-positive cells (monocytes), at a purity of > 90%, from one buffy coat. These cells can be completely converted into DC by adding cytokine.

**Example 2: Cell culture**

The isolated CD14-positive cells were taken up in 12 ml of c-RPMI [(cRPMI: "RPMI 1640" (Gibco, Paisley, Scotland) containing 10% heat-inactivated foetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin); all the constituents contain less than 0.025 international endotoxin unit/ml (guide value for water for injection)]. In order to allow the cells to mature into dendritic cells (DCs), human GM-CSF for clinical use (Leukomax 400®, Sandoz AG, Nuremberg) and IL-4 (Genzyme, Rüsselsheim, batch tested for its LPS content: < 0.001 ng/ml) were added to them at concentrations of 8 000 units/ml of medium and 500 units/ml of medium, respectively. The cell suspension was then pipetted into a six-well plate at the rate of 2 ml per well and cultured in an incubator at 37°C and



5% CO<sub>2</sub>. On the fourth day, a further 2 ml of c-RPMI containing GM-CSF and IL-4 were added to each well.

**Example 3: Hyaluronic acid preparations**

5 a) **Fractionating hyaluronic acid and separating fragments**

Purified cockscomb hyaluronic acid (HA) (Healon®; Pharmacia, intended for clinical use, endotoxin content < 0.001 ng/mg) was first of all  
10 cleaved into relatively large fragments using an ultrasonicator (Branson Sonifier) for 2 min. Hyaluronidase (Type I from bull testes; Sigma) was then added to the fragments in order to break them down still further. The reaction solution was adjusted to  
15 pH 5 with sodium acetate and incubated at 37°C for 12 hrs; the hyaluronidase was then inactivated by heating at 90°C. The resulting fragments were then separated on the basis of their size. For this, they were layered on a polyacrylamide gel (Bio-Gel P-10,  
20 Bio-Rad, Munich) in a 1.5 m-long glass column. Double distilled water was used as the washing liquid, and a fraction collector (Pharmacia) below the column collected fractions every 20 min. The tubes were sealed and stored cool and protected from light.

25 b) **Detecting the sizes of the hyaluronic acid fragments**

1. *Relatively large fragments obtained following ultrasonication size reduction*

Sonicated HA was fractionated by gel  
30 electrophoresis in a 5% agarose gel. The polysaccharide bands in the gel were visualized by staining with Stains-all (3,3'-diethyl-9-methyl-4,5,4',5'-dibenzo-thiacarbocyanine; Sigma). The fragments obtained in this way are from 10 000 to 50 000 kDa in size.

2. *Small fragments obtained following additional digestion with hyaluronidase*

ANTS labelling:

First of all, the individual samples had to be labelled with the fluorophore ANTS [ANTS solution: 0.15 M 8-aminonaphthalene-1,3,6-trisulphonic acid disodium salt in acetic acid/water (3/17, v/v), dissolved by slowly heating to 60°C]. ANTS labels each sugar molecule at the end of the chain. The small fragments were likewise fractionated by gel electrophoresis using a 30% acrylamide gel. It was then possible to visualize the gel, and record it photographically, under UV light (ANTS dye).

c) Quantitatively determining the hyaluronic acid concentration after the fragmentation and separation

0.1 ml was withdrawn from each sample and mixed, in an icebath, with 0.6 ml of staining solution (5.2 g of disodium tetraborate in 1 l of concentrated sulphuric acid). 0.02 ml of 0.1% carbazole in ethanol was then added in each case and the reaction components were mixed and boiled again for 10 min. After the reaction mixtures had cooled down to room temperature, the HA concentration was determined photometrically at 520 nm. Distilled water was used for the blank value, while 0.2  $\mu$ mol of HA/ml in distilled water was used as the standard.

Example 4: Cell stimulation

In order to stimulate the cells, different HA fragments were added to them, at a concentration of 0.025 mg/ml (25  $\mu$ g/ml) of medium, on the fourth day of the culture. Lipopolysaccharides (LPS) obtained from *Escherichia coli* serotype 0177 (Sigma) were used as the positive control.

**Results:**

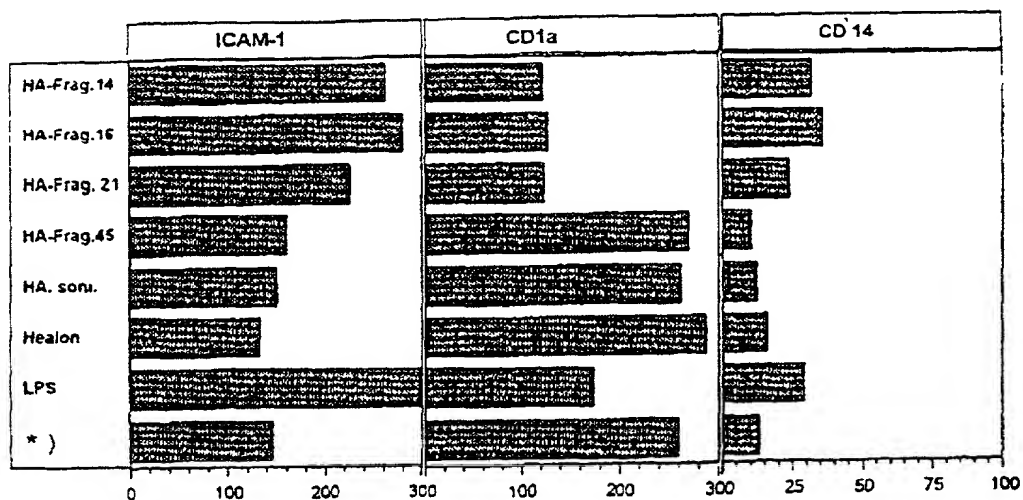
1) Separating the resulting fragments by gel electrophoresis and ANTS staining. The fragments which are used in accordance with the invention are from 2 up

to 12 sugar molecules in size. The concentration measurement gave values of about 1 mg of cleaved HA/ml. In principle, it is possible to cleave this fraction further into individual sugars by means of HPLC (high pressure liquid chromatography). The results lead to the conclusion that it is particularly the small fragments which are responsible for the stimulation.

2) Effect of the stimulation of DC by HA fragments on the expression of surface markers.

Antibodies can be used to establish the surface density of various receptors using the FACS images. Integrating the areas under the curves gives the so-called MFI (mean fluorescence intensity) value. These values are listed for various receptors in Table 1.

Table 1



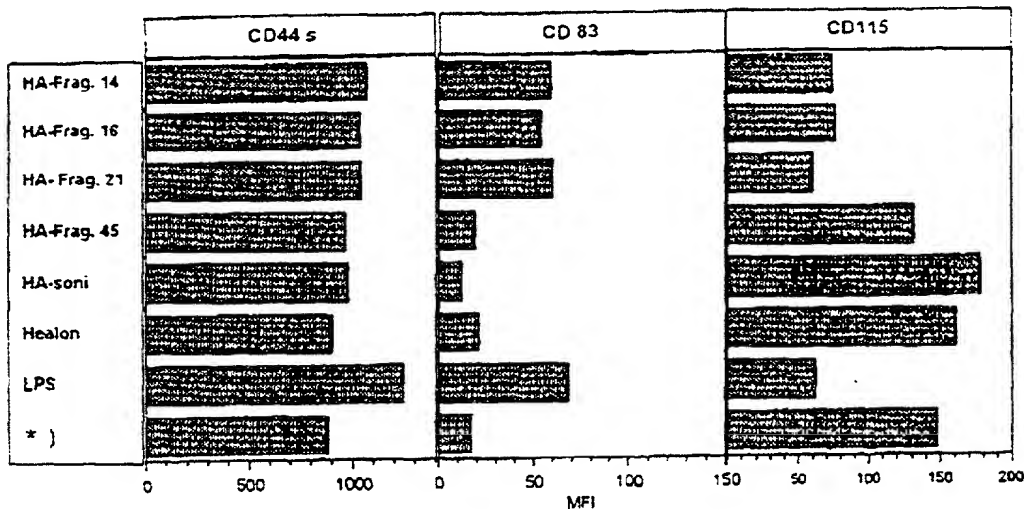
\*) Untreated immature DCs, day 4

Regarding the importance of the individual surface markers cited in Table 1:

ICAM-1 is an intercellular adhesion molecule which occurs almost ubiquitously. The results show a slight up-regulation, as is observed in very many different types of cell activation. CD1a is a marker for dendritic cells and Langerhans cells of the skin;

while monocytes only express CD14, there is an ever increasing loss of CD14, and expression of CD1a, during the course of the maturation. While LPS, HA fragments and MCM medium suppress this process to a slight extent, the functional consequences of this regulation are unknown.

Table 2:

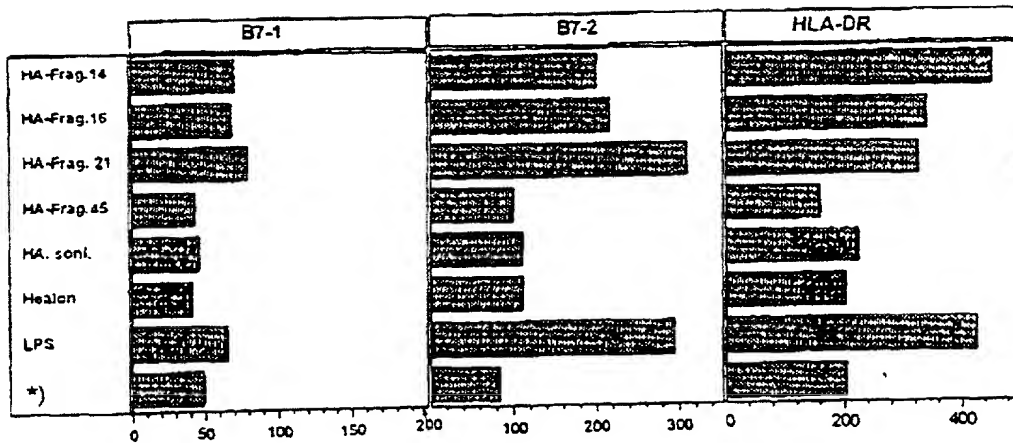


\*) Untreated immature DCs, day 4

Table 2 shows the stimulation of other surface markers (CD44s, CD83 and CD115). While a further sign of DC maturation is a downregulation of CD115 (the G-CSF receptor) and an up-regulation of CD83, the functional relevance of these changes is still not known. It can be clearly seen from Table 2 that the criteria of DC maturation by stimulation with small HA fragments are fully met.

CD44 is an adhesion molecule, a reported function of which is that of binding HA.

Table 3



\*) Untreated immature DCs, day 4

5

Table 3 shows the expression of other surface markers.

This table depicts factors, apart from maturation markers, which play a crucial role for the function of the cells in antigen presentation, i.e. in stimulating T lymphocytes. These factors include the MHC class II molecule HLA-DR and the two costimulatory factors B7-1 and B7-2. Without these factors being up-regulated, there would be no point, either, in using DCs for the above-described applications. According to the invention, a marked up-regulation of all the factors was found after treatment with HA fragments, with this up-regulation being comparable with the maximum stimulation achieved following the addition of LPS.

20

### Example 5

In order to portray the increased expression of surface molecules in a functional manner, the DCs were prestimulated and then incubated for 5 days with purified, naive allogenic T cells which had likewise been obtained from leukocyte concentrate (buffy coats) (so-called mixed leukocyte reaction, MLR). Under these conditions, the T cells proliferated, as measured by the incorporation of radioactive  $^3\text{H}$ -thymidine, with

30

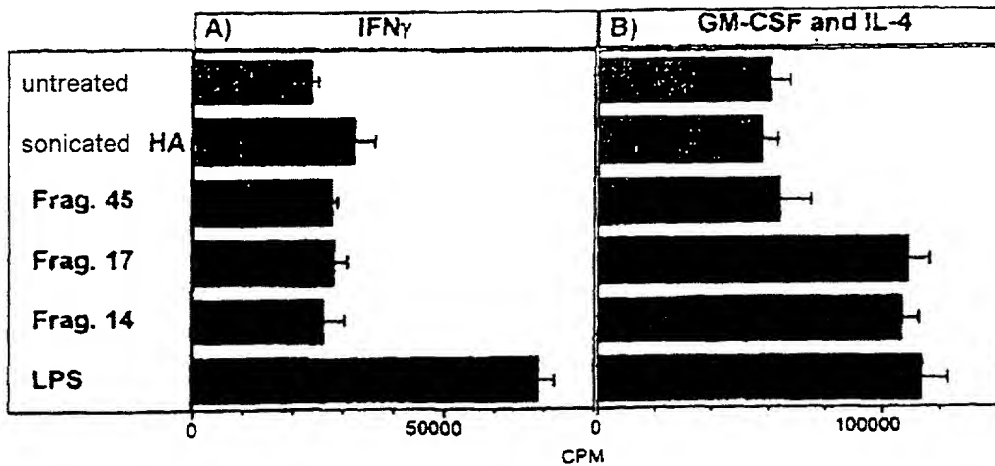
this proliferation being more or less pronounced depending on the stimulatory capacity of the DCs. The radioactive counts per minute (cpm) in the individual samples, which counts correlate directly with the T cell proliferation which occurred, are given in Table 4. It can be seen that DCs which were treated with small HA fragments are markedly more potent APCs, with the values for these DCs being comparable with that obtained for LPS stimulation (shown in Table 4 B).

This example, too, provides evidence that a marked maturation of DCs is obtained when small HA fragments, of between 2 and 12 UDP-sugar molecules, are used in accordance with the invention, with this maturation being entirely comparable with published data from other methods. While strict dependence on a single sugar appears to be unlikely, larger molecules (20-30 UDP-sugars) evidently have no influence since the fractions which are used in accordance with the invention do not exhibit any differences in their effect even when they contain larger fragments. Sonicated HA does not have any effect, either.

#### Example 6

Interestingly, the effect according to the invention is evidently specific for DCs, since gamma interferon (IFN $\gamma$ )-treated monocytes, which mature into macrophages, do not exhibit any increase in their stimulatory capacity following treatment with HA fragments (see Table 4 A).

Table 4



- 5 Frag. 45: No detection of small HA fragments in gel electrophoresis; in the uronic acid assay, less than 0.001 mg of HA = negative fraction for excluding an effect of column constituents.
- 10 Frag. 17: In gel electrophoresis, the largest detectable bands are at approx. 16-fold sugars; sugars having a size of up to 6-fold sugars are strongly represented. Uronic acid assay: 1 mg/ml.
- 15 Frag. 14: Gel electrophoresis: up to approx. 22-fold sugars, strong bands up to 10-fold sugars; uronic acid assay: 1 mg/ml.

#### Example 7

- 20 In this example, it is shown that dendritic cells which have been matured with low molecular weight hyaluronic acid fragments are surprisingly superior to conventional dendritic cells in inducing a peptide-mediated, hapten-specific immunity.
- 25 Mice (C57/BL6, female, 6-12 weeks, 60 animals, 5 mice per experimental group, 20 g per mouse) were injected with dendritic cells which had previously been treated with a synthetically prepared protein having a defined amino acid sequence, i.e. a (SIINF $\text{FEK}^*\text{L}$ ,

SIK\*FEKL; \* = TNP lysine) peptide. The peptide is selected such that it can react directly with the MHC molecule antigen-binding site on the cell surface of antigen-presenting cells; this means that it is presented on the surface of dendritic cells immediately after they have been added. The hapten trinitrophenyl (TNP), which constitutes the antigenic determinant, is in turn coupled to this peptide. This means that a T cell which is activated by the trinitrophenyl-coupled peptide which is presented by the dendritic cell becomes reactive to trinitrophenyl or chemicals which are structurally homologous to trinitrophenyl, such as the trinitrochlorobenzene (TNCB) used during the challenge which is implemented below. Accordingly, in this system, the strength of the trinitrophenyl-specific T cell reaction depends on the degree to which the dendritic cells are activated and/or the efficiency with which the trinitrophenyl-coupled peptide is presented to the T cells by the dendritic cells. The method is a recognized model for the in-vivo investigation of mechanisms which play a role during T cell activation.

Appropriate methods are more precisely described, for example, in the publications J. Invest. Dermatol. 1998, 110: 441-48; Eur. J. Immunol. 1995, 25: 92-101 and J. Immunol. 1993, 151: 678-87.

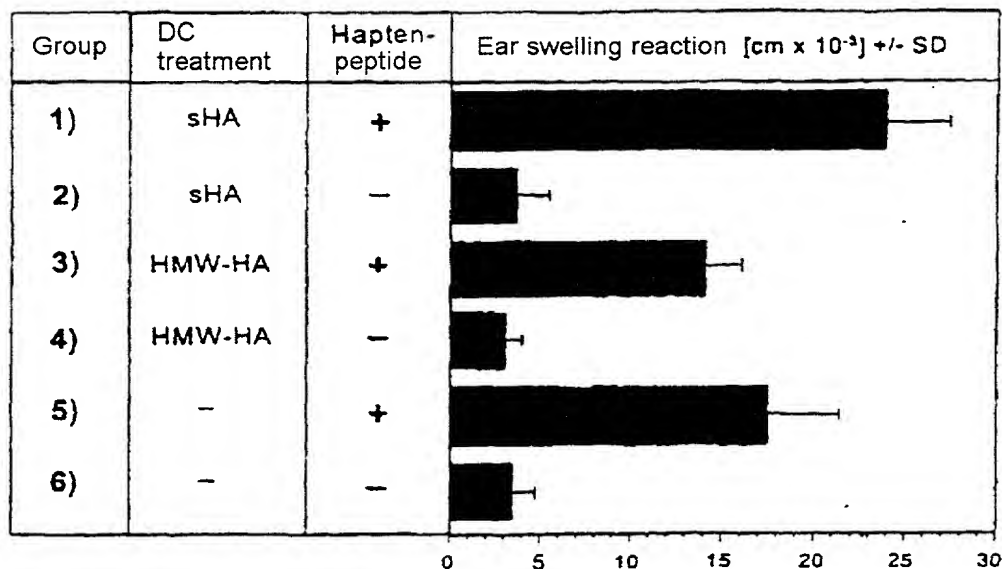
The injected dendritic cells induce a T cell-mediated immune response within a week. That is, after having been injected, they migrate into the lymphatic organs and induce the above-described T cell activation in these organs. After this period of time, the efficiency of the immunization which has been effected can be quantified with the aid of an ear swelling reaction, which is induced by directly applying (painting the ear with a 1% solution of TNCB) a trinitrophenyl analogue, in our case trinitrochlorobenzene (TNCB) to the ear. Within 24 h, this treatment induces an inflammation reaction whose strength depends on the number of memory T cells present in the skin. The ear swelling reaction



is measured as the increase in thickness of the ear in  $\mu\text{m}$ . The results are summarized in Table 5 below.

Table 5

5



In this system, a marked increase in the ear swelling reaction was detected after the dendritic cells had been pretreated with low molecular weight hyaluronic acid fragments. The injection of untreated dendritic cells (group 6) scarcely induces any detectable ear swelling reaction, as is also the case when the dendritic cells are only pretreated with high molecular weight hyaluronic acid (group 4) or low molecular weight hyaluronic acid fragments (group 2). When the dendritic cells are pretreated with hapten-peptide (group 5), a marked ear swelling reaction of approx. 0.15 mm then ensues. The T cell response can be significantly improved by additionally pretreating the dendritic cells with low molecular weight hyaluronic acid fragments, and then displays an ear swelling reaction of approx. 0.24 mm (group 1). On the other hand, pretreating the dendritic cells with high molecular weight hyaluronic acid and hapten-peptide (group 3) does not result in any significant improvement as

compared with the untreated control group 5.

**Example 8**

In this example, vaccinations with dendritic  
5 cells, which had been treated with low molecular weight  
hyaluronic acid fragments, were tested for their  
ability to induce antiviral immunity using a mouse  
model having specificity for lymphocytic chorio-  
meningitis virus (LCMV). The test method employed is  
10 known in principle from the publication J. Virol. 1998,  
72: 3812-18.

In the same manner as described in Example 7,  
dendritic cells were treated, for this purpose, with  
GP-33, a peptide which encodes the antigenic determi-  
15 nant of a surface antigen of the LCM virus, and then  
injected into the mouse intravenously. As previously  
described, this immunizes the animal against LCMV, with  
the efficiency of the immunization depending on the  
degree of activation and/or the pretreatment of the  
20 dendritic cells. The efficiency of the immunization was  
then determined after a week by inoculating the mouse  
with the complete LCMV. Successful immunization against  
the virus can be deduced from a clonal expansion of  
GP-33-specific T cells in the blood and the lymphatic  
25 organs of the treated animals. In another assay, the  
virus load (number of viruses) is determined in the  
peripheral blood.

In a second embodiment, which will be described  
below, the invention makes available a vaccine which  
30 comprises at least one antigen or peptide and a low  
molecular weight hyaluronic acid fragment, which may be  
modified, where appropriate, as an immune response  
amplifier (adjuvant). Surprisingly, it has been found  
that low molecular weight hyaluronic acid fragments,  
35 which may be modified, where appropriate, can advan-  
tageously be used as an adjuvant when directly  
vaccinating with antigens or peptides.

In this method, a vaccine, which comprises the  
antigen, is injected into the patient subcutaneously

(s.c.), intracutaneously (i.c.) or else intravenously (i.v.). The injected antigen is taken up by endogenous antigen-presenting cells (APC), e.g. the dendritic cells, and presented in the regional lymph nodes, thereby inducing an antigen-specific immune response. Surprisingly, it was possible to show that simultaneously injecting low molecular weight hyaluronic acid fragments, which can be chemically modified, where appropriate, amplifies the immune response by recruiting, activating and maturing APC at the puncture site.

The low molecular weight hyaluronic acid fragments, or their chemical modifications, have already been described in detail in the first embodiment of the invention. The low molecular weight hyaluronic acid fragments, or their chemical modifications, which can be employed in this second embodiment of the invention are the same as those described in the first embodiment. They are likewise prepared as described above.

In principle, the novel vaccine according to the second embodiment can comprise any antigen or peptide which is to elicit an immune response. The hyaluronic acid fragments, or the chemical modifications thereof, are preferably present in the same solution in which the antigen or peptide is also present. However, it is also possible to administer the antigen or peptide separately from the low molecular weight hyaluronic acid fragments or their chemical modifications. A vaccine which comprises both the antigen or peptide and the low molecular weight hyaluronic acid fragment, which can be chemically modified, where appropriate, comprises the antigen or peptide at a concentration of from approx. 1 to 1 000 µg/ml, preferably at a concentration of from 10 to 100 µg/ml, and the low molecular weight hyaluronic acid fragment, or its chemical modifications, at a concentration of from 10 ng/ml to 1 000 µg/ml, preferably at a concentration of from 10 µg/ml, to 100 µg/ml, e.g. 30 µg/ml. A suitable daily dose of antigen or

peptide is, for example, from 1 to 1 000 µg/day, preferably from 10 to 100 µg/day, and the daily dose of the low molecular weight hyaluronic acid fragments, or their chemical modifications, can, for example, be from  
5 10 ng/day to 1 000 µg/day, preferably from 10 µg/day to 100 µg/day, e.g. 30 µg/day.

Apart from the antigen or the peptides and the low molecular weight hyaluronic acid fragments, which can be chemically modified, where appropriate, the compositions according to the invention can also comprise  
10 other auxiliary substances which are known in the prior art, e.g. Freund's adjuvant.

According to the invention, the low molecular weight hyaluronic acid fragments, or their chemical  
15 modifications, can be administered and used in a manner which is customary in the field and with which the skilled person is familiar.

The following example explains this second embodiment of the invention.

20

#### Example 9

In order to test the in-vivo activity of low molecular weight hyaluronic acid fragments, healthy test subjects (N = 3) were injected subcutaneously with  
25 100 µl of a 1 mg/ml preparation of low molecular weight hyaluronic acid fragments (one basic unit and fragments containing from 2 to 20 basic units) and, as control, 100 µl of isotonic sodium chloride solution (0.9% NaCl) on two consecutive days. On day three, punch biopsies  
30 were removed and the preparations were worked up immunohistologically. From the clinical point of view, a slight inflammatory reaction, in the form of a discrete reddening around the puncture site, appeared only 24 h after the subcutaneous injection of low  
35 molecular weight hyaluronic acid fragments, as did a marked infiltrate, which mainly consisted of activated, mature dendritic cells. The results obtained with the immunohistological preparations are summarized in Table 6. The cells infiltrating into the corium were

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counted under the microscope, at 40 times magnification, using a grid eyepiece; 3 x 12 fields were counted per preparation and the number of infiltrating cells per mm<sup>2</sup> were calculated from this.

5 Table 6 shows the clear increase in infiltrating cells which took place only 24 h after injecting low molecular weight hyaluronic acid fragments; by contrast, the control injection, which was carried out in parallel, does not display any such effect. The

10 precise immunophenotyping of the infiltrate shows a marked preponderance of CD1a and HLA-DR-positive cells at the sites treated with low molecular weight hyaluronic acid fragments, i.e. the cells display the phenotype of mature dendritic cells. It is not possible

15 to observe any clear increase in lymphocytes or individual subclasses (CD19 = B cell marker, CD3 = T cell marker, CD4 = T helper cell marker, CD8 = labelled cytotoxic T cells) at least in the period over which the observation took place (Table 6).

20 In marked contrast to this finding, the infiltrate obtained following the control injection of isotonic sodium chloride solution shows a substantially more homogeneous distribution, which is comparable to the distribution of the individual subclasses which is

25 found in association with a non-specific inflammatory reaction (Table 6).

Table 6

Period of observation	24 h	24 h	48 h	48 h
Agent	sHA frag.	0.9% NaCl	sHA frag.	0.9% NaCl
Infiltrating cells/mm <sup>2</sup>	365	123	453	235
CD1a-positive	89%	49%	78%	42%
HLA-DR-positive	75%	34%	83%	45%
CD3-positive	23%	44%	28%	52%
CD4-positive	15%	26%	17%	40%
CD8-positive	6%	8%	8%	15%
CD19-positive	2%	1%	1%	3%

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The example provides evidence that, in addition to potentially activating dendritic cells in vitro, in accordance with the first embodiment of the invention, 5 low molecular weight hyaluronic acid fragments are evidently also able to elicit a directed immigration of activated dendritic cells to the injection site in vivo. After having taken up coinjected antigen/peptides, or antigen-peptides which are coupled 10 to low molecular weight hyaluronic acid fragments, these dendritic cells then migrate to the regional lymph nodes, where they are able to induce a specific T cell-mediated immune response.

The third embodiment of the invention, which 15 makes available vaccines which comprise a system in which the low molecular hyaluronic acid fragments, or their chemical modifications, are coupled to an antigen or peptide, is described below. In particular, this embodiment of the invention solves the problem that, 20 while immune responses can be induced by means of a local antigen injection, these responses remain, to a large extent, restricted to the injected limb or even only to the nearest lymph node station. When the novel vaccines according to the third embodiment are used, it 25 is also possible to administer the antigen systemically, e.g. intravenously, without the adjuvant (the low molecular weight hyaluronic acid fragments which can be chemically modified, where appropriate) becoming inactive within a short period of time due to the 30 dilution effect in the peripheral blood.

The low molecular weight hyaluronic acid fragments, or their chemical modifications, are the same as those which have already been described in the first embodiment and can be prepared in the same manner.

35 Antigens or peptides which are customarily employed in vaccines, preferably in vaccines for tumour therapy, may be mentioned as the antigen or peptide, where appropriate together with a carrier system.

The nature of the coupling between the low

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molecular weight hyaluronic acid fragment, which can be chemically modified, where appropriate, and the peptide or antigen, where appropriate together with a carrier system, is not restricted in any particular way. Preference is given to the coupling taking place by the chemical covalent bonding of the antigen or peptide to the adjuvant (low molecular weight hyaluronic acid fragment or chemical modification thereof) or by the antigen or peptide and the adjuvant being jointly included in a microsphere, as is described, for example, in Cancer Res. 1998, 58: 3385-90.

Particular preference is given to coupling the basic building block, UDP- $\beta$ -D-N-acetylglucosamine, which is used in synthesizing hyaluronic acid, to the antigen or, preferably to the peptide. This means that particular preference is given, in the third embodiment of the invention, to the hyaluronic acid fragment which contains one basic unit. As has been previously explained, the basic unit is an aminodisaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by a  $\beta$ 1-3-glycosidic bond. UDP- $\beta$ -D-N-acetylglucosamine is commercially available.

The vaccines in accordance with the third embodiment of the invention can be produced using methods and additives which are, in principle, known in the prior art. The only essential point is that use is made of the novel combinations of the peptide or antigen, where appropriate together with a carrier system, and the low molecular weight hyaluronic acid fragment, which can be chemically modified, where appropriate. It goes without saying that, in the present case, the chemical modification of the hyaluronic acid fragment can also be selected such that a suitable chemical coupling to the antigen or peptide can be effected advantageously. Suitable chemical modifications and coupling methods are known in principle to the skilled person, and any arbitrary methods of the prior art can be used.

A novel vaccine according to the third embodi-

ment of the invention comprises, for example, from 1 µg/ml to 1 000 µg/ml, preferably from 10 µg/ml to 100 µg/ml, of the antigen or peptide, where appropriate together with a carrier system. A suitable daily dose of antigen or peptide, where appropriate together with a carrier system, is, for example, from 1 µg/day to 1 000 µg/day, preferably from 10 µg/day to 100 µg/day. If the coupling between the antigen or peptide and the low molecular weight hyaluronic acid fragment, or chemical modification thereof, has taken place by covalent bonding, the concentration or daily dose of the low molecular weight hyaluronic acid fragment, or of its chemical modification, is necessarily the same as the corresponding concentration or daily dose of the antigen or peptide, where appropriate together with a carrier system. If the low molecular weight hyaluronic acid fragment, which can be chemically modified, where appropriate, and the antigen or peptide are not bonded together chemically but, instead, are jointly included in a microsphere, for example, the preferred concentration of the low molecular weight hyaluronic acid fragment, or its chemical modification, is likewise preferably the same as the concentration of the antigen or peptide.

Possible additives for the vaccine are those additives which are generally customary, e.g. Freund's adjuvant. The vaccine is produced in a manner which is in principle known from the prior art.

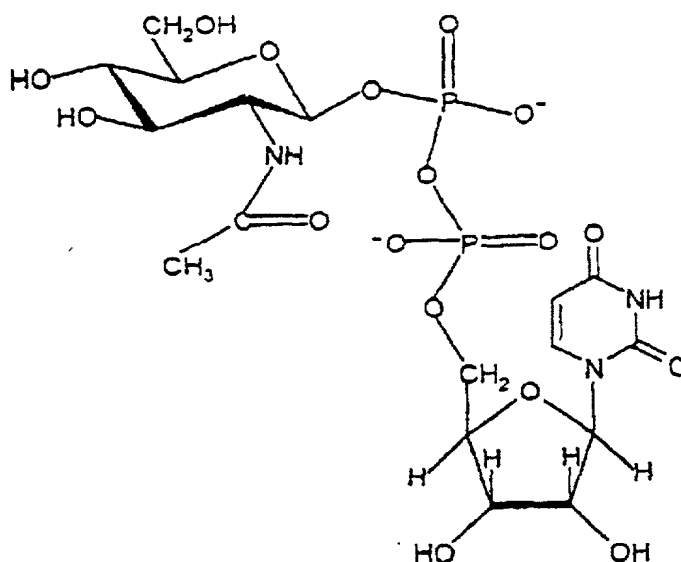
The example explains the third embodiment of the invention.

#### Example 10

In order to bring the vaccine peptide and the adjuvant as close together as possible, the low molecular weight hyaluronic acid fragment, or the basic building block, UDP-β-D-N-acetylglucosamine used in the hyaluronic acid synthesis (fragment containing one basic unit) was coupled directly to the peptide. UDP-β-D-N-Acetylglucosamine can be obtained as a pure



substance from Oxford Glucosciences and exhibits an effect on dendritic cells which is similar to that exhibited by hyaluronic acid cleavage products both as regards the required concentration of the substance and as regards the degree of activation. The well-known conversion of aldehydes and amino groups to Schiff's bases is used for the coupling. A certain percentage of the sugar molecule is present in its open-chain aldehyde form and enters into the abovementioned reaction with the protein in aqueous solution.



UDP- $\beta$ -D-N-Acetylglucosamine

In order to stabilize the product, the compound is reduced by adding sodium borohydride ( $\text{NaBH}_4$ ), thereby forming, with the elimination of water, a covalent bond between the carbon atom of the aldehyde group and the nitrogen atom of the amino group on the peptide.

In order to check its activity, the compound which has been prepared in this way can be administered either intracutaneously or subcutaneously in 50-100  $\mu\text{l}$  of isotonic sodium chloride solution. As previously described, the peptide-HA compound binds to MHC molecules on the surface of antigen-presenting cells,

e.g. dendritic cells, and should simultaneously activate the cells. The compound can also be administered intravenously.

5 Preferred examples of peptides against tumour antigen or virus antigen, which peptides are preferred in all the embodiments according to the invention, are listed in Table 7:

Table 7

10

Antigen name:	Peptide sequence:	Target cell:
MELAN-1/MART-1	EAAGIGILTV	human melanoma
Tyrosinase	AFLPWHRLFL	human melanoma
GP-33	KAVYNFATM	LCM virus

15

The novel vaccines according to all the embodiments can comprise the antigen or peptide, where appropriate together with a suitable carrier system as well. Examples of these carrier systems are liposomes and microsomes, which, as is known from the prior art, can be prepared from phospholipid compounds.

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**Patent Claims**

- 1) Use of low molecular weight hyaluronic acid fragments, which can be suitably modified, where appropriate, for producing a vaccine.
- 2) Use according to Claim 1, in which the vaccine can be used for treating cancer diseases.
- 3) Use according to Claim 1 or 2, in which the hyaluronic acid fragments, which can be suitably modified, where appropriate, consist of from 1 to 50 basic units.
- 4) Use according to Claim 3, in which the hyaluronic acid fragment is UDP- $\beta$ -D-N-acetylglucosamine (fragment from one basic unit).
- 5) Use according to one of Claims 1 to 4, wherein the low molecular weight hyaluronic acid fragments, which can be suitably modified chemically, where appropriate, are used for concentrating dendritic cells which are then employed as the vaccine.
- 6) Process for concentrating dendritic cells, which comprises the following steps:
  - a) mononuclear cells are isolated from blood,
  - b) cells which possess the CD14 surface marker are concentrated,
  - c) the cells which possess the CD14 surface marker are cultured in a medium which contains the cytokines GM-CSF and IL-4, and
  - d) the cells obtained in step c) are cultured together with hyaluronic acid fragments, which can be suitably modified, where appropriate, in order to cause the cells to mature irreversibly into dendritic cells.
- 7) Process according to Claim 6, characterized in that the blood mononuclear cells are isolated from a leukocyte concentrate using a density gradient, in particular a Ficoll density gradient.
- 8) Process according to Claim 6 or 7, charac-

terized in that the cells possessing the CD14 surface marker are concentrated using at least one antibody which is directed against the CD14 surface marker.

9) Process according to one of Claims 6 to 8, characterized in that the cells possessing the CD14 surface marker are cultured in a medium which contains GM-CSF at a concentration of from 5 000 to 10 000 U/ml and IL-4 at a concentration of from 100 to 1 000 U/ml.

10) Process according to one of Claims 6 to 9, characterized in that the cells in step d) are cultured together with hyaluronic acid fragments which contain from 1 to 50 hyaluronic acid basic building blocks, with the basic building block being an amino-disaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine which are linked by a  $\beta$ 1-3 glycosidic bond.

11) Process according to Claim 10, characterized in that the hyaluronic acid fragments each contain from 1 to 10 aminodisaccharides.

12) Process according to one of Claims 6 to 11, characterized in that the cells possessing the CD14 surface marker are cultured, for between 72 hours and 7 days, in a medium containing GM-CSF and IL-4.

13) Process according to one of Claims 6 to 12, characterized in that the cells in step d) are cultured together with hyaluronic acid fragments for at least 48 hours.

14) Process according to one of Claims 6 to 13, characterized in that chemically modified hyaluronic acid fragments are used.

15) Enriched dendritic cells, obtainable by a process according to one of Claims 6 to 14.

16) Use of enriched dendritic cells according to Claim 15 for producing a vaccine.

17) Use according to Claim 16, in which the vaccine is used for treating cancer diseases.

18) Use according to one of Claims 1 to 4, wherein the vaccine comprises low molecular weight hyaluronic acid fragments, which can be suitably modified

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chemically, where appropriate, and an antigen or peptide, where appropriate together with a carrier system.

5 19) Vaccine comprising an antigen or peptide, where appropriate together with a carrier system and a low molecular weight hyaluronic acid fragment which can be suitably modified chemically, where appropriate.

20) Vaccine according to Claim 19, wherein the vaccine is prepared for subcutaneous, intracutaneous or  
10 intravenous administration.

21) Vaccine according to Claim 19 or 20, wherein the vaccine consists of a formulation which comprises the antigen or peptide, where appropriate together with a carrier system, and the low molecular weight  
15 hyaluronic acid fragment, which can be suitably modified, where appropriate.

22) Vaccine according to Claim 19 or 20, wherein the vaccine comprises two separate formulations, with one formulation comprising the antigen or peptide,  
20 where appropriate together with a carrier system, and the other formulation comprising the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate.

23) Vaccine according to one of Claims 19 to 22 for  
25 use in treating cancer diseases.

24) Vaccine according to one of Claims 19 to 23 wherein the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, consists of from 1 to 50 basic units.

30 25) Use according to one of Claims 1 to 4, wherein the vaccine comprises a system in which the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, is coupled to a peptide or antigen.

35 26) System comprising a low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, coupled to an antigen or peptide.

27) System according to Claim 26, in which the low

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molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, is chemically bonded to the antigen or peptide.

28) System according to Claim 26, in which the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, and the peptide or antigen are present as separate molecules in a microsphere.

29) System according to one of Claims 26 to 28, in which the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, comprises from 1 to 50 basic building blocks.

30) System according to Claim 29, in which the low molecular weight hyaluronic acid fragment, which can be suitably modified, where appropriate, is UDP- $\beta$ -D-N-acetylglucosamine (fragment from one basic building block).

31) System according to one of Claims 26 to 30, in which the antigen or peptide is present together with a carrier system.

32) Vaccine comprising a system according to one of Claims 26 to 31.

33) Vaccine according to Claim 32 for treating cancer diseases.

Albert Ludwigs University  
Clinical Centre, Freiburg

**Abstract**

The invention discloses the use of low molecular weight hyaluronic acid fragments, which can be suitably modified, where appropriate, for producing vaccines. The vaccines are particularly suitable for controlling cancer diseases. Surprisingly, it has been found that low molecular weight hyaluronic acid fragments, which can be suitably modified, where appropriate, can be used both for preparing mature dendritic cells and directly, together with antigens or peptides or carrier systems, as an adjuvant in vaccines. It is likewise possible to couple the low molecular weight hyaluronic acid fragments, which can be suitably modified, where appropriate, to an antigen, peptide or carrier system. The coupled system can then be advantageously employed as a vaccine, in particular for treating cancer diseases.

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**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name:

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**LOW MOLECULAR WEIGHT FRAGMENTS OF HYALURONIC ACID FOR  
PRODUCING VACCINES**

the specification of which (check one)

☐ is attached hereto.

☒ was filed on **August 26, 1999** United States Application Serial No. or PCT International Application No. **PCT/EP99/06280** and was amended on \_\_\_\_\_ by Preliminary Amendment, (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand that contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

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I HEREBY CLAIM foreign priority benefits under Title 35, United States Code §119(a)-(d) of §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claim?	Certified Copy Attached?
19839113.7	GERMANY	August 27, 1998	Yes	No
19853066.8	GERMANY	November 17, 1998	Yes	No

I HEREBY CLAIM the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

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U.S. Patent Application Number	PCT Patent Application Number	Patent Filing Date	Parent Patent Number

I HEREBY APPOINT the following registered attorneys and agents of the law firm of Heller Ehrman White & McAuliffe LLP to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith:

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John P. Isacson	Reg. No. 33,715
Ronald J. Kamis	Reg. No. 41,104
Susan E. Shaw McBee	Reg. No. 39,294
Marvin Motsenbocker	Reg. No. 36,614
Colin G. Sandercock	Reg. No. 31,298

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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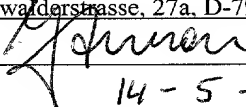
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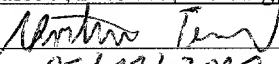
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1-00 09763764-071201  
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